



## University of Groningen

### 100% complete assignment of non-labile H-1, C-13, and N-15 signals for calcium-loaded calbindin D-9k P43G

Oktaviani, Nur Alia; Otten, Renee; Dijkstra, Klaas; Scheek, Ruud M.; Thulin, Eva; Akke, Mikael; Mulder, Frans A. A.

*Published in:*  
Biomolecular nmr assignments

*DOI:*  
[10.1007/s12104-010-9272-3](https://doi.org/10.1007/s12104-010-9272-3)

**IMPORTANT NOTE:** You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

*Document Version*  
Publisher's PDF, also known as Version of record

*Publication date:*  
2011

[Link to publication in University of Groningen/UMCG research database](#)

*Citation for published version (APA):*

Oktaviani, N. A., Otten, R., Dijkstra, K., Scheek, R. M., Thulin, E., Akke, M., & Mulder, F. A. A. (2011). 100% complete assignment of non-labile H-1, C-13, and N-15 signals for calcium-loaded calbindin D-9k P43G. *Biomolecular nmr assignments*, 5(1), 79-84. <https://doi.org/10.1007/s12104-010-9272-3>

#### Copyright

Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

#### Take-down policy

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

*Downloaded from the University of Groningen/UMCG research database (Pure): <http://www.rug.nl/research/portal>. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.*

# 100% complete assignment of non-labile $^1\text{H}$ , $^{13}\text{C}$ , and $^{15}\text{N}$ signals for calcium-loaded calbindin $\text{D}_{9\text{k}}$ P43G

Nur Alia Oktaviani · Renee Otten · Klaas Dijkstra ·  
Ruud M. Scheek · Eva Thulin · Mikael Akke ·  
Frans A. A. Mulder

Received: 20 August 2010 / Accepted: 24 September 2010 / Published online: 12 November 2010  
© The Author(s) 2010. This article is published with open access at Springerlink.com

**Abstract** Here we present the 100% complete assignment chemical shift of non-labile  $^1\text{H}$ ,  $^{15}\text{N}$  and  $^{13}\text{C}$  nuclei of Calbindin  $\text{D}_{9\text{k}}$  P43G. The assignment includes all non-exchangeable side chain nuclei, including ones that are rarely reported, such as  $\text{LysN}_\zeta$  as well as the termini. NMR experiments required to achieve truly complete assignments are discussed. To the best of our knowledge our assignments for Calbindin  $\text{D}_{9\text{k}}$  extend beyond previous studies reaching near-completeness (Vis et al. in Biochem 33:14858–14870, 1994; Yamazaki et al. in J Am Chem Soc 116:6464–6465, 1994; Yamazaki et al. in Biochem 32:5656–5669, 1993b).

**Keywords** Calbindin  $\text{D}_{9\text{k}}$  · 100% Complete assignment · Assignment strategy · NMR spectroscopy

## Biological context

Calbindin  $\text{D}_{9\text{k}}$  is a small monomeric protein (Mr 8.5 kDa, 76 amino acids) which belongs to the EF-hand family, and consists of two helix-loop-helix motifs that bind one calcium ion each (Kretsinger and Nockolds 1973). Calbindin  $\text{D}_{9\text{k}}$  undergoes small structural changes upon calcium-binding, involving the rearrangement of non-polar side

chains (Ikura 1996). The protein is predominantly found in the mammalian epithelial cells of the small intestine and placenta, and it has been implicated to facilitate the transport of calcium across the intestinal epithelial cells (Christakos et al. 1989).

High resolution three dimensional structures of Calbindin  $\text{D}_{9\text{k}}$  in various calcium-loaded states have been characterized extensively by X-ray crystallography and solution state NMR spectroscopy (Kordel et al. 1997; Kordel et al. 1993; Szebenyi and Moffat 1986). Although the complete resonance assignment of  $^{13}\text{C}$ ,  $^{15}\text{N}$  and  $^1\text{H}$  nuclei for this protein was not used for structure determination, it will facilitate a comprehensive study of its dynamics, structure, dihedral angle distributions and electrostatic interactions, as well as supplying data for comparisons with solid-state NMR and chemical shift calculations by quantum chemical methods.

## Methods and experiments

MM294 *E. coli* cells transformed by the PCBWR plasmid containing the *calbindin* Pro43Gly gene (*Bos taurus*) were used for protein expression. A single colony was picked from agar plate and grown overnight in 100 mL LB medium with ampicillin at 30°C. 20 mL of the overnight culture was added to 500 mL minimal medium containing  $\text{U-}^{13}\text{C}$ -glucose and  $\text{U-}^{15}\text{N}$  ammonium chloride at 30°C. Protein production was started by ten-fold dilution of the cells into medium containing  $\text{U-}^{13}\text{C}$  glucose,  $\text{U-}^{15}\text{N}$  ammonium chloride and 0.1 mg/mL IPTG at 37°C. Purification of Calbindin  $\text{D}_{9\text{k}}$  P43G was performed as in a previous study (Thulin 2002).

All experiments (see Table 1) were carried out on Varian Unity INOVA 500 and 600 MHz spectrometers

N. A. Oktaviani · R. Otten · K. Dijkstra ·  
R. M. Scheek · F. A. A. Mulder (✉)  
Groningen Biomolecular Sciences and Biotechnology Institute,  
University of Groningen, Nijenborgh 4, 9747 AG Groningen,  
The Netherlands  
e-mail: f.a.a.mulder@rug.nl

E. Thulin · M. Akke · F. A. A. Mulder  
Department of Biophysical Chemistry, Lund University,  
PO Box 124, Lund, Sweden

**Table 1** List of experiments

No	Experiments	Connectivities	Experimental time (h)
1	$^1\text{H}-^{15}\text{N}$ HSQC-SE-wfb <sup>a,b,c</sup>	$\text{N}^{\text{H}}-\text{H}^{\text{N}}$	0.2
2	Sensitivity-enhanced HA(CA)CO <sup>d,e</sup>	$\text{H}\alpha-\text{C}'$	0.6
3	3D-HN(C')N <sup>f</sup>	$\text{N}_{(i)}^{\text{H}}-\text{H}_{(i)}^{\text{N}}-\text{N}_{(i+1)}^{\text{H}}$	20.5
4	3D-HNN <sup>f</sup>	$\text{N}_{(i)}^{\text{H}}-\text{H}_{(i)}^{\text{N}}-\text{N}_{(i)}^{\text{H}}$	55.8
		$\text{N}_{(i)}^{\text{H}}-\text{H}_{(i)}^{\text{N}}-\text{N}_{(i)}^{\text{H}}$	
		$\text{N}_{(i)}^{\text{H}}-\text{H}_{(i)}^{\text{N}}-\text{N}_{(i-1)}^{\text{H}}$	
		$\text{N}_{(i)}^{\text{H}}-\text{H}_{(i)}^{\text{N}}-\text{N}_{(i+1)}^{\text{H}}$	
5	H(N)CO <sup>g</sup>	$\text{H}_{(i)}^{\text{N}}-\text{C}'_{(i-1)}$	0.3
6	HACA(N) <sup>h</sup>	$\text{H}\alpha_{(i)}-\text{C}\alpha_{(i)}$	1.75
7	H2(C)N <sup>i</sup>	$\text{N}^{\text{H}}-\text{H}\alpha$	0.3
8	H2(CA)N <sup>i</sup>	$\text{N}\zeta-\text{H}\epsilon(\text{Lysine})$	0.6
		$\text{N}-\text{H}\alpha(\text{Proline})$	
9	3D $^1\text{H}-^{15}\text{N}$ -TOCSY-HSQC <sup>j,k,l,m</sup>	Nterminus-H $\alpha$	8.25
10	3D HCCH-COSY <sup>n,o</sup>	$\text{N}_{(i)}^{\text{H}}-\text{H}_{(i)}^{\text{N}}$ -all aliphatic side chain protons <sub>(i)</sub>	17
11	3D C-TOCSY-N(C)H2 <sup>i</sup>	$\text{C}_{(i)}-\text{H}_{(i)}-\text{H}_{(i)}$ (through one bond coupling of aliphatic resonances)	20
12	3D H(CCO)NH-TOCSY	$\text{H}\epsilon-\text{N}\epsilon$ and all side chain carbons of lysine	21.25
13	3D H(C(CO)NH-TOCSY <sup>q</sup>	$\text{N}_{(i)}^{\text{H}}-\text{H}_{(i)}^{\text{N}}$ -all aliphatic side chain protons <sub>(i-1)</sub>	14
14	$^1\text{H}-^{13}\text{C}$ constant time HSQC <sup>r</sup>	$\text{N}_{(i)}^{\text{H}}-\text{H}_{(i)}^{\text{N}}$ -all aliphatic side chain carbons <sub>(i-1)</sub>	0.2
15	(HBGCBG)CO(CBGCABCON)H <sup>t</sup>	$\text{C}_{(i)}-\text{H}_{(i)}$ of aliphatic resonances	4
16	H2(C)CO <sup>u</sup>	$\text{C}_{\gamma(i)}-\text{H}_{(i+1)}^{\text{N}}$ for asparagine and aspartic acid	2
		$\text{C}\delta_{(i)}-\text{H}_{(i+1)}^{\text{N}}$ for glutamine and glutamatic acid	
		$\text{C}'-\text{H}\alpha$	
		$\text{C}_{\gamma}-\text{H}\beta$ (for asparagine and aspartate)	
17	$^3\text{J}_{\text{NC}\gamma}^s$	$\text{C}\delta-\text{H}\gamma$ (for glutamate and glutamine)	8.6
		$\text{C}_{\gamma(i)}-\text{H}_{(i)}^{\text{N}}$	
		$\text{C}\beta_{(i)}-\text{H}_{(i)}^{\text{N}}$	
		$\text{C}\alpha_{(i)}-\text{H}_{(i)}^{\text{N}}$	
18	$^3\text{J}_{\text{C}'\text{C}\gamma}^s$	$\text{C}\alpha_{(i-1)}-\text{H}_{(i)}^{\text{N}}$ (if i-1 is glycine)	2.16
		$\text{C}_{\gamma(i)}-\text{H}\epsilon 2_{(i)}$ for glutamine	
		$\text{C}\beta_{(i)}-\text{H}\delta 2_{(i)}$ for asparagine	
		$\text{C}'_{(i-1)}-\text{H}_{(i)}^{\text{N}}$	
		$\text{C}_{\gamma(i-1)}-\text{H}_{(i)}^{\text{N}}$	
		$\text{C}\beta_{(i)}-\text{H}_{(i)}^{\text{N}}$	
19	3D $^1\text{H}-^{13}\text{C}$ HSQC NOESY <sup>v</sup>	$\text{C}\beta_{(i-1)}-\text{H}_{(i)}^{\text{N}}$	38
		$\text{C}\alpha_{(i-1)}-\text{H}_{(i)}^{\text{N}}$ (for proline)	
20	3D HCCH-COSY aromatic <sup>o,p</sup>	$\text{C}_{(i)}-\text{H}_{(i)}$ -all protons within 5 Å	16.8
21	CG(CB)HB <sup>w</sup>	C-H-H	9.3
22	CB(CGCD)HD <sup>x</sup>	$\text{C}_{\gamma}-\text{H}\beta$ for aromatic side chain	10.8
23	CB(CGCDCE)HE <sup>x</sup>	$\text{C}\beta-\text{H}\delta$ for aromatic side chain	10.8
24	$^1\text{H}-^{13}\text{C}$ HSQC aromatic <sup>r</sup>	$\text{C}\beta-\text{H}\epsilon$ for aromatic side chain	0.8
		$\text{C}\delta-\text{H}\delta$	
		$\text{C}\epsilon-\text{H}\epsilon$	
		$\text{C}\zeta-\text{H}\zeta$ for aromatic side chain	

**Table 1** continued

No	Experiments	Connectivities	Experimental time (h)
25	$^1\text{H}$ - $^{13}\text{C}$ HSQC CP aro <sup>y</sup>	C $\delta$ -H $\delta$ C $\delta$ -H $\epsilon$ C $\epsilon$ -H $\epsilon$ C $\epsilon$ -H $\zeta$ C $\zeta$ -H $\zeta$ for aromatic side chain	7
26	$^1\text{H}$ - $^{13}\text{C}$ HMQC aromatic <sup>z</sup>	C $\delta$ -H $\delta$ C $\epsilon$ -H $\epsilon$ C $\zeta$ -H $\zeta$ for aromatic side chain	0.3

<sup>a</sup> Cavanagh et al. 1991; <sup>b</sup> Palmer et al. 1991; <sup>c</sup> Palmer et al. 1992; <sup>d</sup> Kay et al. 1990b; <sup>e</sup> Powers et al. 1991; <sup>f</sup> Panchal et al. 2001; <sup>g</sup> Muhandiram and Kay 1994; <sup>h</sup> Ottiger and Bax 1997; <sup>i</sup> Andre et al. 2007; <sup>j</sup> Fesik and Zuiderweg 1990; <sup>k</sup> Marion et al. 1989a; <sup>l</sup> Marion et al. 1989b; <sup>m</sup> Zhang et al. 1994; <sup>n</sup> Ikura et al. 1991; <sup>o</sup> Kay et al. 1990a; <sup>p</sup> Ikura et al. 1991; <sup>q</sup> Logan et al. 1993; <sup>r</sup> Vuister and Bax 1992; <sup>s</sup> Konrat et al. 1997; <sup>t</sup> Tollinger et al. 2002; <sup>u</sup> Oda et al. 1994; <sup>v</sup> Majumdar and Zuiderweg 1993; <sup>w</sup> Prompers et al. 1998; <sup>x</sup> Yamazaki et al. 1993a; <sup>y</sup> Zuiderweg et al. 1996; <sup>z</sup> Bax et al. 1990

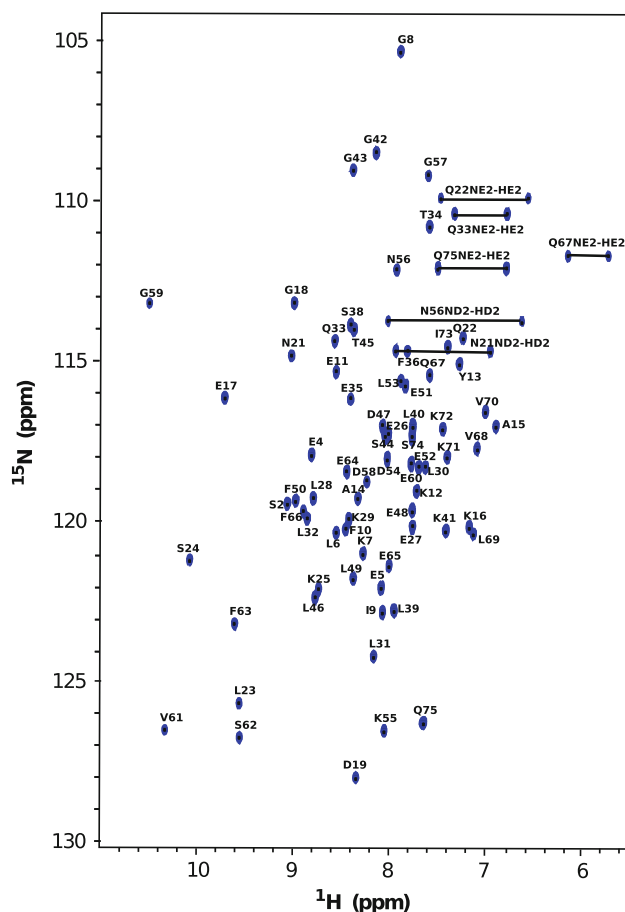
equipped with pulsed field gradient probes. The spectra were recorded at 301 K. The NMR sample contained  $\sim 2.5$  mM [ $^{13}\text{C}$ ,  $^{15}\text{N}$ ]-enriched Calbindin D<sub>9k</sub> P43G, pH 6.0, 7% D<sub>2</sub>O. The spectra were processed using NMRPipe (Delaglio et al. 1995) and analyzed using Sparky (Goddard and Kneller 2003).

### Assignments and data deposition

Relation to previous assignment (BMRB entry 327): Only  $^1\text{H}$  signals of Calbindin D<sub>9k</sub> P43G had been assigned. Here the 100% complete assignment of non-labile  $^1\text{H}$ ,  $^{13}\text{C}$  and  $^{15}\text{N}$  signals for calcium-loaded D<sub>9k</sub> P43G was achieved using an extensive suit of standard and non-standard 2D and 3D NMR experiments.

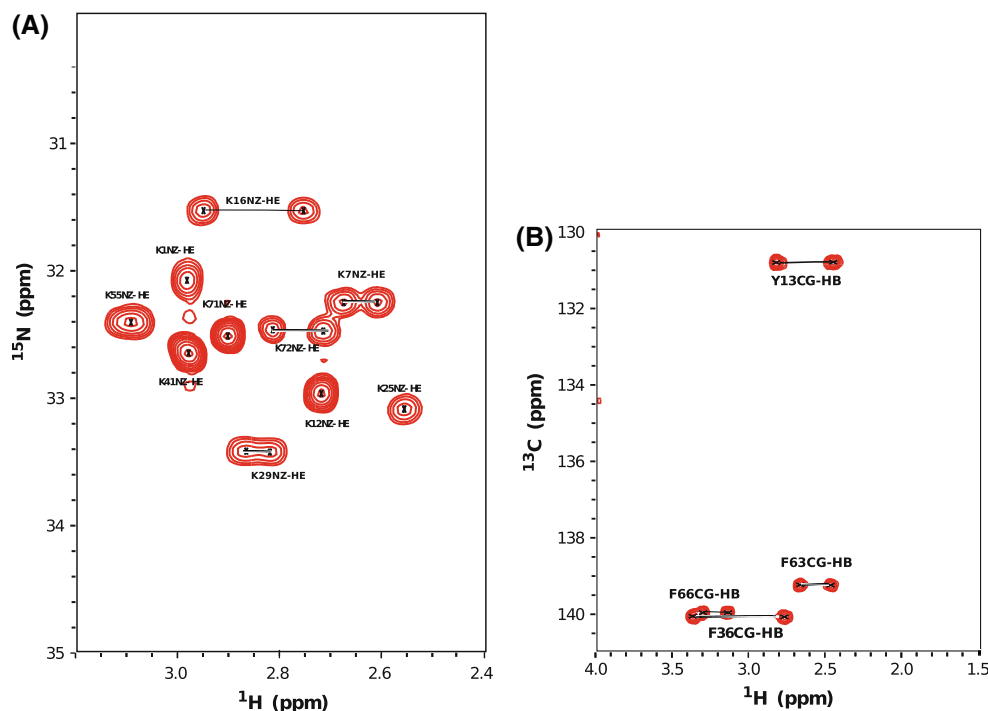
### Backbone and aliphatic side chains

Backbone assignments were obtained using 3D experiments such as HN(C')N and HNN to obtain the amide proton and nitrogen chemical shifts (Fig. 1 displays the dispersion of  $\text{N}^{\text{H}}\text{-H}^{\text{N}}$  chemical shift of Calbindin D<sub>9k</sub> from  $^1\text{H}$ - $^{15}\text{N}$  HSQC). Several 2D projections of triple resonance experiments; H(N)CO, HA(CA)CO, and HACA(N) were recorded to assign the carbonyl, alpha proton and alpha carbon resonances. Most aliphatic side chain signals were assigned using 3D experiments; (H)C(CO)NH-TOCSY and H(CCO)NH-TOCSY, which correlate the backbone nitrogen and amide proton shifts to aliphatic carbon and proton frequencies of residue  $i - 1$ . For residues which are preceded by proline, the side chain nuclei were assigned using a 3D  $^{15}\text{N}$ - $^1\text{H}$  TOCSY-HSQC experiment which correlates



**Fig. 1** 2D  $^1\text{H}$ - $^{15}\text{N}$  HSQC spectrum of uniformly  $^{15}\text{N}/^{13}\text{C}$ -labelled Calbindin D<sub>9k</sub>. All peaks are annotated with the one letter amino acid symbol and their position in the sequence. All amide proton and nitrogen nuclei in the backbone of Calbindin D<sub>9k</sub> were observed. The  $^{15}\text{N}_\epsilon$  and  $^1\text{H}_\epsilon$  chemical shifts of glutamine and the chemical shifts of  $^{15}\text{N}_\delta$  and  $^1\text{H}_\delta$  of asparagines are also indicated

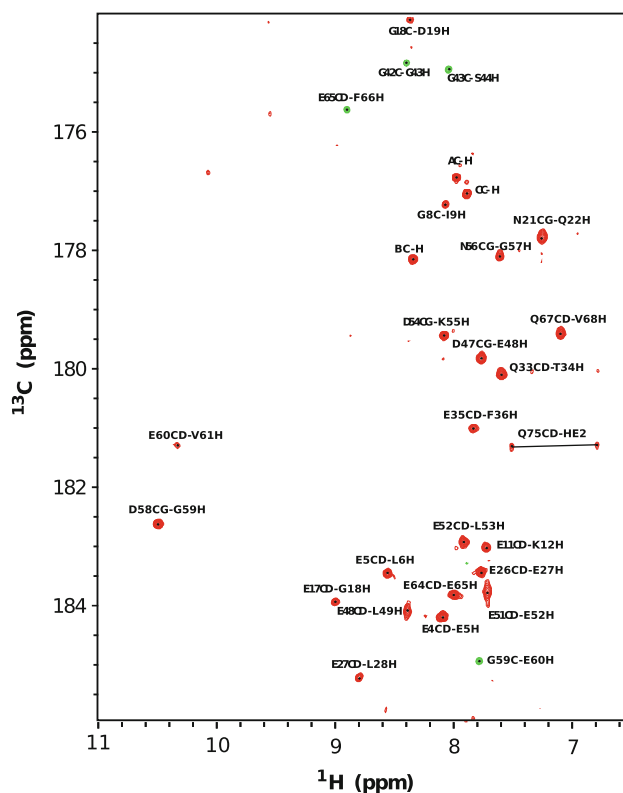
**Fig. 2** Some specific assignments of Calbindin D<sub>9k</sub> side chains. **a** 2D H<sub>2</sub>(C)N spectrum showing Lys N $\zeta$ -H $\epsilon$  correlations. **b** 2D CG(CB)HB spectrum to assign aromatic side chain resonances



$N^H$ ,  $H^N$ , and proton aliphatic side chain in the same residue. The HCCH-COSY experiment, which gives information about chemical shifts of protons bound to carbon, enabled us to verify the assignment for long side chains like lysine, leucine, glutamate, isoleucine, valine and proline. The specific assignment of lysine N $\zeta$  were obtained using the 2D H<sub>2</sub>(C)N experiment. The signals in this spectrum are well dispersed between 31 and 34 ppm (see Fig. 2a). The  $^{15}N$  shift of the N terminal methionine residue was obtained using 2D H<sub>2</sub>(C)N pulse sequence where the final shaped carbon inversion pulse was replaced by a full power rectangular 180° pulse (Andre et al. 2007). The carbonyl side chains of glutamate, glutamine, asparagine and aspartate were detected using (HBGCBG)CO(CBGCABCACON)H experiments which correlates the side chain carbonyl of residue *i* to the amide proton of residue *i*+1 (see Fig. 3). This experiment is very powerful to get the unambiguous chemical shift assignment of carbonyl/carboxyl side chains due to the excellent dispersion of amide proton chemical shifts in folded proteins. However, the sensitivity of the experiments was not sufficient to detect all signals (Q22 was absent) and a H<sub>2</sub>(C)CO experiment was used to detect the Q22 C $\delta$ -H $\gamma$  correlation.

#### Aromatic side chains

Calbindin D<sub>9k</sub> P43G contains 5 phenylalanines and 1 tyrosine residue. Some specific strategies were required to assign the aromatic ring  $^1H$  and  $^{13}C$  resonances. Aromatic C $\gamma$  resonances were assigned using a combination of CG(CB)HB,



**Fig. 3** 2D  $^1H$ - $^{13}C$  (HBGCBG)CO(CBGCABCACON)H spectrum of uniformly labelled  $^{15}N/^{13}C$  Calbindin D<sub>9k</sub>. Correlation can be observed for carboxyl/carbonyl side chain  $^{13}C'$  of glutamate, glutamine, asparagine and aspartate of residue *i* with the amide proton of residue *i*+1. Peaks labeled AC-H, BC-H and CC-H refer to carbonyl and amide proton peaks from the soluble cyclic enterobacterial common antigen, ECA<sub>CYC</sub> (Erbel et al. 2003)

$^3\text{J}_{\text{NC}\gamma}$  and  $^3\text{J}_{\text{C}'\text{C}\gamma}$  experiments. The sequence-specific side chain  $^1\text{H}$  assignment of the aromatic side chains was obtained via CB(CGCD)HD and CB(CGCDCE)HE experiments. The information of H $\delta$  and H $\epsilon$  in the aromatic rings from these experiments were used to assign  $^1\text{H}$ – $^{13}\text{C}$  HSQC CP aro, aromatic  $^1\text{H}$ – $^{13}\text{C}$  CT HSQC and  $^1\text{H}$ – $^{13}\text{C}$  HMQC aromatic experiment. F10 C $\delta$ –H $\delta$  was only observed in the non constant time  $^1\text{H}$ – $^{13}\text{C}$  HSQC or  $^1\text{H}$ – $^{13}\text{C}$  HMQC experiment due to the strong coupling within the aromatic ring. A 3D  $^1\text{H}$ – $^{13}\text{C}$  HSQC-NOESY experiment was used to verify the assignment of the aromatic side chains.

To summarize, the  $^1\text{H}$ ,  $^{13}\text{C}$  and  $^{15}\text{N}$  resonance assignments of Calbindin D<sub>9k</sub> P43G have been deposited in the BioMagResBank (accession number 16340). Although complete side chain resonance assignments were obtained for Calbindin D<sub>9k</sub> P43G, it should be mentioned that it does not contain any cysteine, arginine, histidine and tryptophan residues. Those residues, in particular, require specific strategies for their side chain assignment. For arginine guanidine groups, sequence specific assignment of  $^{15}\text{N}$  and  $^1\text{H}$  chemical shifts have been presented by Yamazaki et al. (1995), and for histidine and tryptophan ring, sequence specific assignment of  $^1\text{H}$ ,  $^{13}\text{C}$ ,  $^{15}\text{N}$  have been established by Löhr et al. (2005).

Our study shows that complete assignments of all NMR-active nuclei in small protein can be obtained and describes a suitable strategy for this purpose. In particular, lysine N $\zeta$  chemical shifts appear to be difficult to get correct, as witnessed by the BMRB database. Currently (grid update of August 16th, 2010) 110 chemical shift assignments for lysine N $\zeta$  are available, but this list contains as many as 25 erroneous assignments. In three cases, a  $^1\text{H}$  chemical shift was entered for N $\zeta$  and in 22 instances chemical shifts between 67 and 133 ppm have been listed, either as a result of exchanging the assignment with that of backbone nuclei, or by the incorrect account of spectral aliasing. Even today, the lysine N $\zeta$  statistics are heavily polluted and yield  $47.8 \pm 32.9$  ppm for the full set. A restricted set of 14 entries now gives  $34.1 \pm 3.0$  ppm, as opposed to  $73.8 \pm 50.3$  ppm for 7 entries in 2004.

**Acknowledgments** We thank Eldon Ulrich at the BioMagRes Bank for helpful discussion.

**Open Access** This article is distributed under the terms of the Creative Commons Attribution Noncommercial License which permits any noncommercial use, distribution, and reproduction in any medium, provided the original author(s) and source are credited.

## References

Andre I, Linse S, Mulder FAA (2007) Residue-specific pKa determination of lysine and arginine side chains by indirect

- $^{15}\text{N}$  and  $^{13}\text{C}$  NMR spectroscopy: application to apo calmodulin. *J Am Chem Soc* 129:15805–15813
- Bax A, Ikura M, Kay LE, Torchia DA, Tschudin R (1990) Comparison of different modes of two-dimensional reverse-correlation nmr for the study of proteins. *J Magn Reson* 86:304–318
- Cavanagh J, Palmer AG, Wright PE, Rance M (1991) Sensitivity improvement in proton-detected two-dimensional heteronuclear relay spectroscopy. *J Magn Reson* 91:429–436
- Christakos S, Gabrielides C, Rothen WB (1989) Vitamin D-dependent calcium binding proteins: chemistry, distribution, functional considerations, and molecular biology. *Endocr Rev* 10:3–26
- Delaglio F, Grzesiek S, Vuister GW, Zhu G, Pfeifer J, Bax A (1995) NMRpipe—a multidimensional spectral processing system based on unix pipes. *J Biomol NMR* 6:277–293
- Erbel PJA, Barr K, Gao N, Gerwig GJ, Rick PD, Gardner KH (2003) Identification and biosynthesis of cyclic enterobacterial common antigen in escherichia coli. *J Bacteriol* 185:1995–2004
- Fesik SW, Zuiderweg ERP (1990) Heteronuclear three-dimensional nmr-spectroscopy of isotopically labeled biological macromolecules. *Q Rev Biophys* 23:97–131
- Goddard TD, Kneller DG (2003) SPARKY 3. University of California, San Francisco
- Ikura M (1996) Calcium binding and conformational response in EF-hand proteins. *Trends in Biochem Sci* 21:14–17
- Ikura M, Kay LE, Bax A (1991) Improved three-dimensional  $^1\text{H}$ – $^{13}\text{C}$ – $^1\text{H}$  correlation spectroscopy of a  $^{13}\text{C}$ -labeled protein using constant-time evolution. *J Biomol NMR* 1:299–304
- Kay LE, Ikura M, Bax A (1990a) Proton proton correlation via carbon carbon couplings—a three-dimensional NMR approach for the assignment of aliphatic resonances in proteins labeled with carbon-13. *J Am Chem Soc* 112:888–889
- Kay LE, Ikura M, Tschudin R, Bax A (1990b) Three-dimensional triple-resonance NMR-spectroscopy of isotopically enriched proteins. *J Magn Reson* 89:496–514
- Konrat R, Muhandiram DR, Farrow NA, Kay LE (1997) Pulse schemes for the measurement of  $^3\text{J}_{\text{C}'\text{C}\gamma}$  and  $^3\text{J}_{\text{NC}\gamma}$  in  $^{15}\text{N}$ ,  $^{13}\text{C}$  uniformly labeled proteins. *J Biomol NMR* 9:409–422
- Kordel J, Skelton NJ, Akke M, Chazin WJ (1993) High-resolution solution structure of calcium-loaded calbindin-D<sub>9k</sub>. *J Mol Biol* 231:711–734
- Kordel J, Pearlman DA, Chazin WJ (1997) Protein solution structure calculations in solution: solvated molecular dynamics refinement of calbindin D9 k. *J Biomol NMR* 10:231–243
- Kretsinger RH, Nockolds CE (1973) Carp muscle calcium-binding protein: II. Structure determination and general description. *J Biol Chem* 248:3313–3326
- Logan TM, Olejniczak ET, Xu RX, Fesik SW (1993) A general-method for assigning NMR-spectra of denatured proteins using 3D HC(CO)NH-TOCSY triple resonance experiments. *J Biomol NMR* 3:225–231
- Löhr F, Rogov VV, Shi M, Bernhard F, Dötsch V (2005) Triple-resonance methods for complete resonance assignment of aromatic protons and directly bound heteronuclei in histidine and tryptophan residues. *J Biomol NMR* 32:309–328
- Majumdar A, Zuiderweg ERP (1993) Improved  $^{13}\text{C}$ -Resolved HSQC-NOESY spectra in  $\text{H}_2\text{O}$ , using pulsed-field gradients. *J Magn Reson Series B* 102:242–244
- Marion D, Driscoll PC, Kay LE, Wingfield PT, Bax A, Gronenborn AM, Clore GM (1989a) Overcoming the overlap problem in the assignment of proton nmr spectra of larger proteins by use of three-dimensional heteronuclear proton and nitrogen hartmann-hahn multiple quantum coherence and nuclear overhauser multiple quantum coherence spectroscopy—application to interleukin 1. *Biochemistry* 28:6150–6156

- Marion D, Kay LE, Sparks SW, Torchia DA, Bax A (1989b) Three dimensional heteronuclear NMR of nitrogen-15 labeled proteins. *J Am Chem Soc* 111:1515–1517
- Muhandiram DR, Kay LE (1994) Gradient-enhanced triple-resonance 3-dimensional NMR experiments with improved sensitivity. *J Magn Reson Series B* 103:203–216
- Oda Y, Yamazaki T, Nagayama K, Kanaya S, Kuroda Y, Nakamura H (1994) Individual ionization-constants of all the carboxyl groups in ribonuclease HI from *Escherichia-Coli* determined by NMR. *Biochemistry* 33:5275–5284
- Ottiger M, Bax A (1997) An empirical correlation between amide deuterium isotope effects on  $^{13}\text{C}\alpha$  chemical shifts and protein backbone conformation. *J Am Chem Soc* 119:8070–8075
- Palmer AG, Cavanagh J, Wright PE, Rance M (1991) Sensitivity improvement in proton-detected two-dimensional heteronuclear correlation NMR-spectroscopy. *J Magn Reson* 93:151–170
- Palmer AG, Fairbrother WJ, Cavanagh J, Wright PE, Rance M (1992) Improved resolution in three-dimensional constant-time triple resonance NMR spectroscopy of proteins. *J Biomol NMR* 2:103–108
- Panchal SC, Bhavesh NS, Hosur RV (2001) Improved 3D triple resonance experiments, HNN and HN(C)N, for HN and 15 N sequential correlations in ( $^{13}\text{C}$ ,  $^{15}\text{N}$ ) labeled proteins: application to unfolded proteins. *J Biomol NMR* 20:135–147
- Powers R, Gronenborn AM, Clore GM, Bax A (1991) 3-Dimensional triple-resonance NMR of  $^{13}\text{C}/^{15}\text{N}$  enriched proteins using constant-time evolution. *J Magn Reson* 94:209–213
- Prompers JJ, Groenewegen A, Hilbers CW, Pepermans HAM (1998) Two-dimensional NMR experiments for the assignment of aromatic side chains in  $^{13}\text{C}$ -labeled proteins. *J Magn Reson* 130:68–75
- Szebenyi DM, Moffat K (1986) The refined structure of vitamin D-dependent calcium-binding protein from bovine intestine. Molecular details, ion binding, and implications for the structure of other calcium-binding proteins. *J Biol Chem* 261:8761–8777
- Thulin E (2002) Purification of recombinant calbindin  $\text{D}_{9\text{k}}$ . Humana Press inc, Totowa
- Tollinger M, Forman-Kay JD, Kay LE (2002) Measurement of side-chain carboxyl pKa values of glutamate and aspartate residues in an unfolded protein by multinuclear NMR spectroscopy. *J Am Chem Soc* 124:5714–5717
- Vis H, Boelens R, Mariani M, Stroop R, Vorgias CE, Wilson KS, Kaptein R (1994)  $^1\text{H}$ ,  $^{13}\text{C}$ , and  $^{15}\text{N}$  resonance assignments and secondary structure analysis of the HU protein from *Bacillus stearothermophilus* using two- and three-dimensional double- and triple-resonance heteronuclear magnetic resonance spectroscopy. *Biochemistry* 33:14858–14870
- Vuister GW, Bax A (1992) Resolution enhancement and spectral editing of uniformly  $^{13}\text{C}$  enriched proteins by homonuclear broad-band  $^{13}\text{C}$  decoupling. *J Magn Reson* 98:428–435
- Yamazaki T, Forman-Kay JD, Kay LE (1993a) 2-Dimensional NMR experiments for correlating carbon- $^{13}\text{C}$  and proton- $^1\text{H}$  chemical-shifts of aromatic residues in  $^{13}\text{C}$ -labeled proteins via scalar couplings. *J Am Chem Soc* 115:11054–11055
- Yamazaki T, Yoshida M, Nagayama K (1993b) Complete assignments of magnetic resonances of ribonuclease-H from *escherichia coli* by double-resonance and triple-resonance 2D and 3D NMR spectroscopies. *Biochemistry* 32:5656–5669
- Yamazaki T, Lee W, Revington M, Mattiello DL, Dahlquist FW, Arrowsmith CH, Kay LE (1994) An HNCA pulse scheme for the backbone assignment of  $^{15}\text{N}$ ,  $^{13}\text{C}$ ,  $^2\text{H}$ -labeled proteins—application to a 37-kDa trp repressor DNA complex. *J Am Chem Soc* 116:6464–6465
- Yamazaki T, Pascal SM, Singer AU, Forman-Kay JD, Kay LE (1995) NMR pulse scheme for the sequence-specific assignment of arginine guanidino  $^{15}\text{N}$  and  $^1\text{H}$  chemical shifts in proteins. *J Am Chem Soc* 117:3556–3564
- Zhang OW, Kay LE, Olivier JP, Forman-Kay JD (1994) Backbone  $^1\text{H}$  and  $^{15}\text{N}$  resonance assignments of the N-terminal Sh3 Domain of drk in folded and unfolded states using enhanced-sensitivity pulsed-field gradient NMR techniques. *J Biomol NMR* 4:845–858
- Zuiderweg ERP, Zeng L, Brutscher B, Morshauser RC (1996) Band-selective hetero- and homonuclear cross-polarization using trains of shaped pulses. *J Biomol NMR* 8:147–160